Activation of neutrophils and monocytes by a leukocyte-depleting filter used throughout cardiopulmonary bypass

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Objective: Cardiopulmonary bypass elicits systemic inflammation. Depletion of circulating leukocytes might alleviate inflammatory response. We studied the effects of a leukocyte-depleting filter on phagocyte activation during cardiopulmonary bypass.

Methods: Fifty patients undergoing coronary artery bypass grafting were randomly allocated into an arterial line leukocyte filter group (n = 25) with a Pall LeukoGuard 6 leukocyte-depleting filter (LG6; Pall Biomedical, Portsmouth, United Kingdom) and a control group without any filter (n = 25). Blood sampling took place from arterial line at predetermined time points. In the filter group, the sample was taken immediately before the filter; to evaluate activation at the site, an additional sample was taken immediately after the filter. CD11b/CD18 and L-selectin expressions and basal production of hydrogen peroxide were determined with whole-blood flow cytometry, and plasma lactoferrin level was determined with enzyme-linked immunosorbent assay.

Results: Neutrophil CD11b expression was higher in the filter group than in the control group ($P < .001$). Likewise, monocyte CD11b expression, neutrophil hydrogen peroxide production, and lactoferrin plasma levels were all significantly higher, whereas neutrophil and monocyte counts and neutrophil L-selectin expression were all significantly lower in the filter group (all $P < .001$). At 5 minutes of CPB, CD11b expression increased across the filter on neutrophils (median difference 197 relative fluorescence units, range 45-431 relative fluorescence units, $P < .001$) and monocytes (median difference 26 relative fluorescence units, range −68-111 relative fluorescence units, $P < .001$).

Conclusion: The LG6 arterial line leukocyte filter is ineffective in its principal task of diminishing phagocyte activation during cardiopulmonary bypass.

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Cardiopulmonary bypass (CPB) is associated with the development of systemic inflammation[1] characterized by the systemic release of proinflammatory cytokines and activation of the endothelium and phagocytes (neutrophils and monocytes).[2] During CPB, complement-derived C5a mediates the early phase of systemic inflammation.[3] Concomitantly, aortic crossclamping results in myocardial ischemia, followed by endothelial activation during reperfusion.[1] The activated endothelium promotes phagocyte adherence, manifested as rolling and firm adhesion, mediated by selectins and β_2-integrins, followed by emigration of phagocytes in postcapillary venules.[2] Phagocyte activation itself is designated by increases in cell surface integrin CD11b/CD18 expression and concomitant decreases in L-selectin cell surface density.[2]

Sequestered phagocytes release proteolytic enzymes and reactive oxygen species, causing microvascular damage and organ dysfunction.[4] In experimental CPB, postbypass myocardial and pulmonary functions improve consistently when a leukocyte-depleting arterial line filter is used to reduce the number of circulating neutrophils.[5-9] According to in vitro studies, such filters eliminate mainly neutrophils,[10] particularly activated ones.[11] In clinical studies, however, the results have been ambiguous. Although some improvements in the variables studied have been reported,[12-17] Mihaljevic and colleagues[18] found that the use of a leukocyte filter was in fact associated with poor postoperative oxygenation and high plasma levels of elastase, an activation marker of neutrophils. Scholz and associates[19] have reported increased neutrophilic degranulation when using leukocyte filters.

To date, the effect of the leukocyte-depleting filter on the development of systemic inflammation during clinical CPB has not been explored systematically. We hypothesized that the leukocyte filter, by increasing the amount of unphysiologic surface of the extracorporeal circuit, would aggravate phagocyte activation. To address this question, we studied markers of systemic inflammation (phagocyte CD11b and L-selectin expressions, intracellular hydrogen peroxide production, and plasma lactoferrin level) during CPB with and without a leukocyte-depleting arterial line filter. Furthermore, paired blood samples across the filter were taken to evaluate phagocyte activation within the filter.

TABLE 1. Patient characteristics and clinical outcomes

	Control group	Leukocyte filter group
Patients (No.)	25	25
Male	18	20
Female	7	5
Age (y, median and range)	64 (50-78)	66 (51-73)
Patients with previous acute MI (No.)	11	11
Number of affected coronary arteries		
Two-vessel disease (No.)	5	2
Three-vessel disease (No.)	20	23
LVEF before surgery (median and range)	60% (40-83%)	55% (40-83%)
Duration of CPB (min, median and range)	90 (62-130)	83 (51-154)
Duration of aortic crossclamping (min, median and range)	62 (34-99)	58 (28-112)
Pao_2/FIo_2 ratio before extubation (mm Hg, median and range)	261 (150-568)	294 (153-426)
Time to extubation (min, median and range)	524 (302-865)	475 (345-757)
Postop CK-MB (U/L, median and range)	30 (5-265)	24 (5-190)
Postop weight gain (kg, median and range)	3.2 (−0.7-7.3)	3.5 (−1.5-10.4)
ICU stay (d, median and range)	1 (1-5)	1 (1-5)
Hospital stay (d, median and range)	7 (5-17)	7 (6-13)
Postop stroke (No.)	0	1
Postop mortality (%)	0	0

MI, Myocardial infarction; *LVEF,* left ventricular ejection fraction; *Pao_2,* arterial oxygen tension; *FIo_2,* inspired oxygen fraction, *CK,* creatine kinase; *ICU,* intensive care unit.

Materials and Methods

Patients

The study was approved by the institutional ethics committee. Before surgery, informed consent was obtained from each patient. Patients with significant valvular dysfunction or any renal, liver or pancreatic disease were excluded. Patients undergoing elective coronary artery bypass grafting were randomly allocated into two groups: the filter group (n = 25) with a Pall LeukoGuard 6 leukocyte-depleting filter (LG6; Pall Biomedical, Portsmouth, United Kingdom) used during CPB and the control group (n = 25) with no arterial line filter.

Anesthesia and CPB

Routine medications were terminated the evening before the operation, except for β-blockers and long-acting nitrates. Aspirin was halted 1 week before surgery. Total anesthesia with intravenous propofol, alfentanil, and rocuronium was standardized. Outside the range of propofol and alfentanil infusion rates, hemodynamic control was provided by bolus doses of nitroglycerin, ephedrin, or norepinephrine. All patients received 20 mg/kg tranexamic acid (Caprilon; Leiras Inc, Turku, Finland) before CPB. Aprotinin and steroids were not used.

In all patients, a membrane oxygenator (CombactFlo; Dideco SpA, Mirandola, Italy) was used. In the filter group, we used a leukocyte-depleting arterial line filter (LG6) that combines both a 40-μm polyester screen and leukocyte-depleting filtration media. In the control group, no arterial line filter was used. The extracorporeal circuit was primed with 2000 mL Ringer acetate solution. Before cannulation, patients received 300 IU/kg heparin (Heparin Leo; Leo Pharma AB, Malmö, Sweden). The activated clotting time was kept above 480 seconds with additional heparin boluses. All surgical procedures were performed with moderate hypothermia (nasopharyngeal temperature 33°C-34°C). Cold crystalloid cardioplegic solution was used. During CPB, pump flow was kept at 2.4 L/(min · m^2), and perfusion pressure was kept between 50 and 80 mm Hg. Hemofiltration was not used. Before separation from CPB, all patients were rewarmed (nasopharyngeal temperature 37°C and bladder temperature >36°C). After venous decannulation, 1.2 mg protamine sulfate (Protamin-sulfat; Leo-Pharma AS, Oslo, Norway) per 100 units of heparin used was given as an infusion. Suction blood and the volume remaining in the CPB circuit were reinfused.

Blood Sampling

In all patients, blood samples were taken at the following time points: after the induction of anesthesia but before surgery, imme-

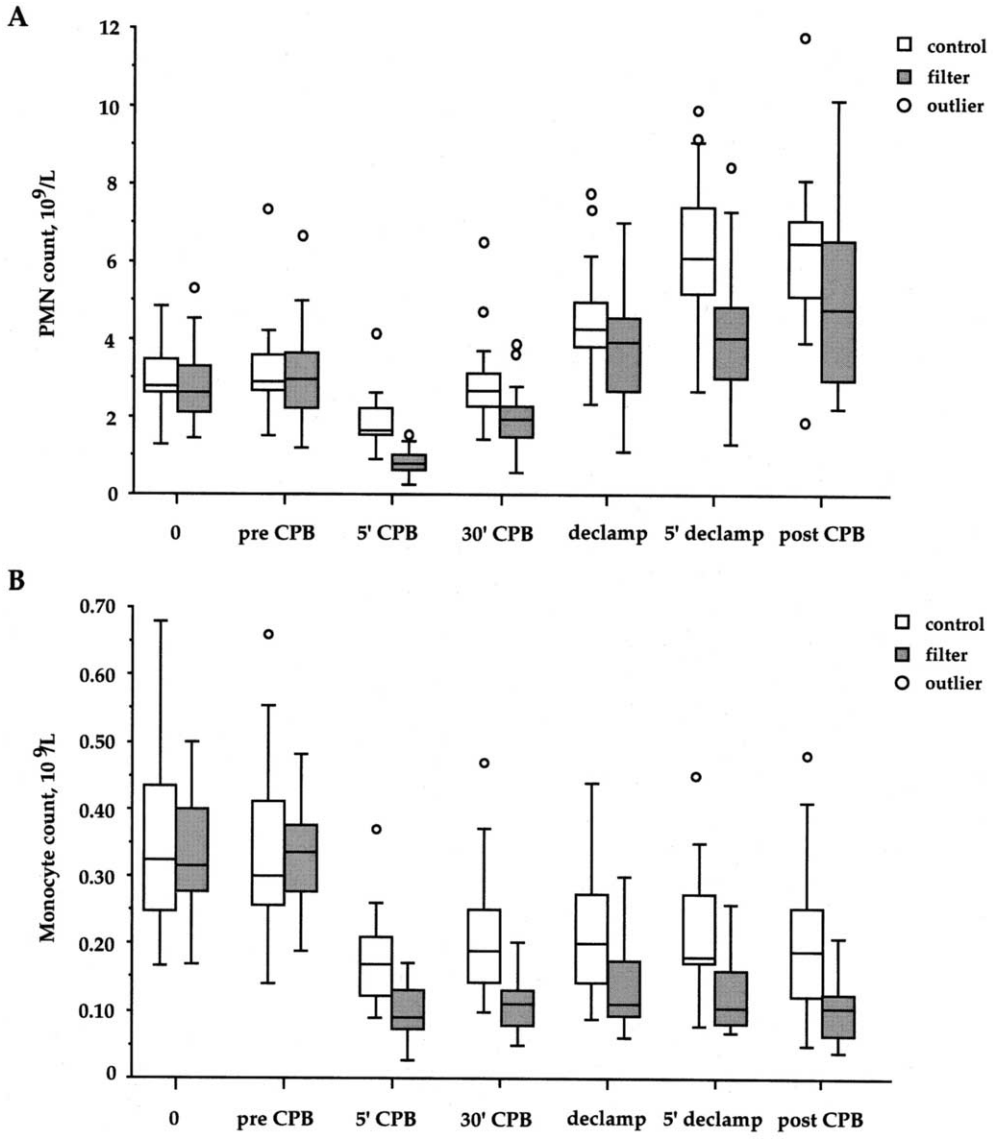

Figure 1. Neutrophil (*PMN,* A) and monocyte (B) counts in control and filter groups. *Boxes* indicate 25th and 75th percentiles and *central lines* indicate median, with *whiskers* representing range. *0,* Before surgery; *pre CPB,* immediately before CPB; *5′ CPB,* 5 minutes of CPB; 30′ CPB, 30 minutes of CPB; *declamp,* immediately before aortic declamping; *5′ declamp,* 5 minutes after aortic declamping, *post CPB,* 5 minutes after CPB. For both neutrophil and monocyte counts, $P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA.

diately before CPB, 5 and 30 minutes after the onset of CPB, immediately before aortic declamping, 5 minutes after aortic declamping, and 5 minutes after cessation of CPB but before protamine administration. In all patients, arterial blood samples were drawn from radial arterial cannula before and after CPB. During CPB, blood samples were drawn from arterial line of the extracorporeal circuit. In the filter group, corresponding samples were drawn simultaneously before and after the filter mounted in the arterial line. The volume of each blood sample was 5 mL. For analysis of leukocyte adhesion molecules, 3 mL of each blood sample was transferred into a tube (Falcon No. 2058; Becton Dickinson Labware, Lincoln Park, NJ) containing 500 μL citrate (Baxter Health Care, Norfolk, United Kingdom), and a 75-μL aliquot was removed for labeling with monoclonal antibodies. The rest of citrated blood was centrifuged at 4°C for 10 minutes at 1000*g,* and plasma was separated and stored at −70°C for lactoferrin determination. For analysis of neutrophil hydrogen peroxide production, 1 mL of each blood sample was incubated in the dark at 37°C for 15 minutes in a polystyrene tube supplemented with 175 μL citrate and 2 μL of 120-μL/mL 2,7-dichlorofluorescein-diacetate (DCFH-DA; Eastman Kodak Company, Rochester, NY). Thereafter, the tube was stored at 0°C in an ice-water bath in the dark until further handling. Finally, 1 mL of each blood sample was transferred into an ethylenediaminetetraacetic acid tube (Va-

TABLE 2. Differences across the filter (after filter minus before filter) during CPB

	5 min CPB	30 min CPB	At declamping	5 min after declamping
Cell counts (10^9 cells/L)				
PMNs	0.01 (−0.26-0.08)	−0.01 (−0.32-0.13)*	−0.04 (−0.43-0.24)	−0.03 (−1.27-0.37)
Monocytes	0.01 (−0.05-0.03)	0.00 (−0.04-0.31)	−0.01 (−0.05-0.04)	0.00 (−0.05-0.09)
CD11b expression (RFU)				
PMN	197 (45-431)*	17 (−62-157)	0 (−61-64)	−6 (−41-66)
Monocytes	26 (−68-111)*	−3 (−165-215)	−4 (−133-70)	12 (−247-111)
L-selectin expression (RFU)				
PMN	−8 (−26-49)†	1 (−227-32)	1 (−53-26)	0 (−9-14)
Monocytes	−4 (−92-51)	−6 (−75-33)	3 (−35-138)	1 (−33-241)
PMN hydrogen peroxide production (RFU)	50 (−324-440)	32 (−125-1315)	14 (−48-936)†	−4 (−84-1057)
Plasma lactoferrin level (g/L)	39 (−117-350)	65 (−896-750)	−11 (−1875-1857)	−170 (−4359-2104)

Data are expressed as medians with ranges. *PMN,* Neutrophil (polymorphonuclear leukocyte).
*$P < .05$, †$P < .01$ after filter versus before filter, Wilcoxon signed rank test.

cutainer Systems Europe, Becton Dickinson, Plymouth, United Kingdom) and stored at room temperature for analysis of leukocyte differential counts (Advia 120; Bayer, Terrytown, NY).

Laboratory Assays

All steps from blood sampling to cell staining were carried out at 0°C in an ice-water bath to minimize artifactual increases in adhesion molecule expression caused by sample handling.[20] Neutrophil and monocyte CD11b and L-selectin (CD62L) expressions and basal intracellular hydrogen peroxide production were determined by flow cytometry as previously described.[20,21] Adhesion molecule expression and 2'-7'-dichlorofluorescein (DCF) fluorescence (intracellular hydrogen peroxide production) are reported as relative fluorescence units (RFU), the mean channel number of the positively fluorescing cell population. Plasma concentration of lactoferrin was measured by enzyme-linked immunosorbent assay.[22]

Statistical Analysis

Because the data distribution was skewed in the filter group according to the Kolmogorov-Smirnov test, a nonparametric approach was instituted. The Mann-Whitney *U* test was used to compare the groups, and the Wilcoxon signed rank test was used for paired comparisons across the filter (after filter minus before filter). To evaluate time-dependent changes within the groups and the interaction between the groups, 1-way repeated measures analysis of variance (ANOVA) was used after appropriate logarithmic transformation of skewed variables. Data are given as medians with ranges.

Results

Filter and control groups were comparable with respect to preoperative patient characteristics, durations of extracorporeal perfusion and aortic crossclamping, and postoperative recovery (Table 1).

Neutrophil and Monocyte Counts

Before surgery, neutrophil counts were comparable in the groups (Figure 1, *A*). Compared with the preoperative level, at 5 minutes of CPB, the neutrophil count had decreased in both groups, but more so in the filter group (median 0.82×10^9 cells/L, range $0.24\text{-}1.75 \times 10^9$ cells/L) than in the control group (median 1.67×10^9 cells/L, range $0.93\text{-}4.22 \times 10^9$ cells/L, $P < .001$, Mann-Whitney *U* test). In both groups, the count increased toward the end of CPB, but it remained lower in the filter group ($P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA, Figure 1, *A*). At 30 minutes of CPB, a marginal yet statistically significant decrease in neutrophil count occurred across the filter (Table 2).

Preoperative monocyte counts were comparable in the groups (Figure 1, *B*). At 5 minutes of CPB, monocyte count had decreased in both groups, but more so in the filter group. Thereafter, monocyte count remained lower in the filter group ($P < .001$ between the groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA, Figure 1, *B*). There was no difference in the monocyte count across the filter (Table 2).

Neutrophil and Monocyte CD11b Expression

Before surgery, neutrophil CD11b expression was comparable in the groups (Figure 2, *A*). In the control group, neutrophil CD11b expression gradually increased during CPB (Figure 2, *A*), whereas in the filter group, CD11b expression abruptly increased during the initial minutes of CPB and at 5 minutes of CPB was already higher (median 795 RFU, range 359-1235 RFU) than in the control group (median 239 RFU, range 185-450 RFU, $P < .001$, Mann-Whitney *U* test). Neutrophil CD11b expression peaked at 30 minutes but remained higher in the filter group throughout CPB ($P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA; Figure 2, *A*). At 5 minutes of CPB, an increase in neutrophil CD11b expression occurred across the filter (median difference 197 RFU,

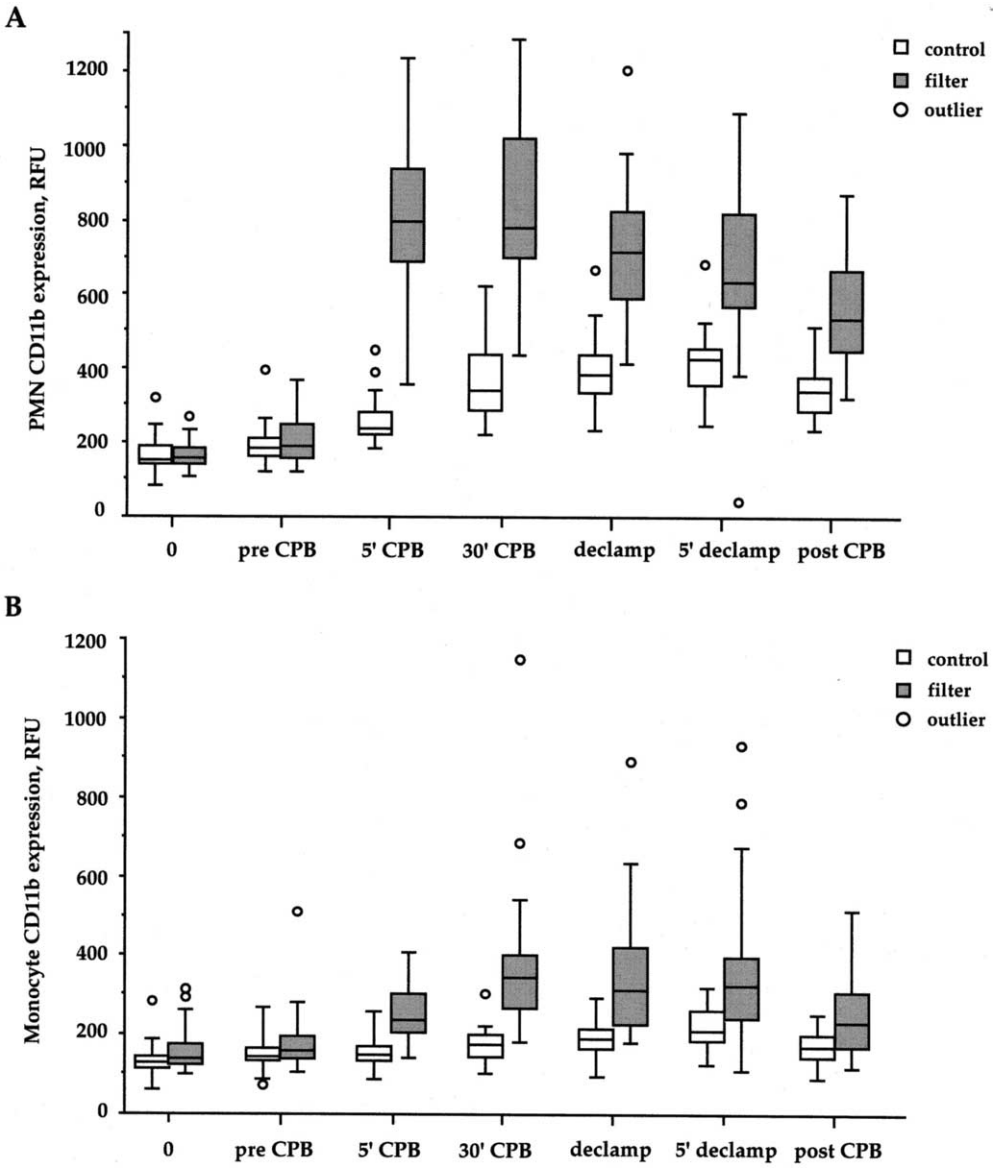

Figure 2. Neutrophil (A) and monocyte (B) CD11b expressions in control and filter groups. *Boxes* indicate 25th and 75th percentiles and *central lines* indicate median, with *whiskers* representing range. *0,* Before surgery; *pre CPB,* immediately before CPB; *5′ CPB,* 5 minutes of CPB; 30′ CPB, 30 minutes of CPB; *declamp,* immediately before aortic declamping; *5′ declamp,* 5 minutes after aortic declamping, *post CPB,* 5 minutes after CPB. For both neutrophil and monocyte CD11b expressions, $P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA.

range 45-431 RFU, $P < .001$, Wilcoxon signed rank test; Table 2).

Preoperative monocyte CD11b expression was comparable in the groups (Figure 2, *B*). At 5 minutes of CPB, monocyte CD11b expression was already higher in the filter group (median 238 RFU, range 143-407 RFU) than in the control group (median 151 RFU, range 89-257 RFU, $P < .001$, Mann-Whitney *U* test), and remained high thereafter ($P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA; Figure 2, *B*) At 5 minutes of CPB, an increase in monocyte CD11b expression occurred across the filter (median difference 26 RFU, range −68-111 RFU, $P < .001$, Wilcoxon signed rank test; Table 2).

Neutrophil and Monocyte L-selectin Expression

Before surgery, neutrophil L-selectin expression was comparable in the groups (Figure 3, *A*). During CPB, neutrophil L-selectin expression increased gradually in the control group, whereas the expression in the filter group remained at

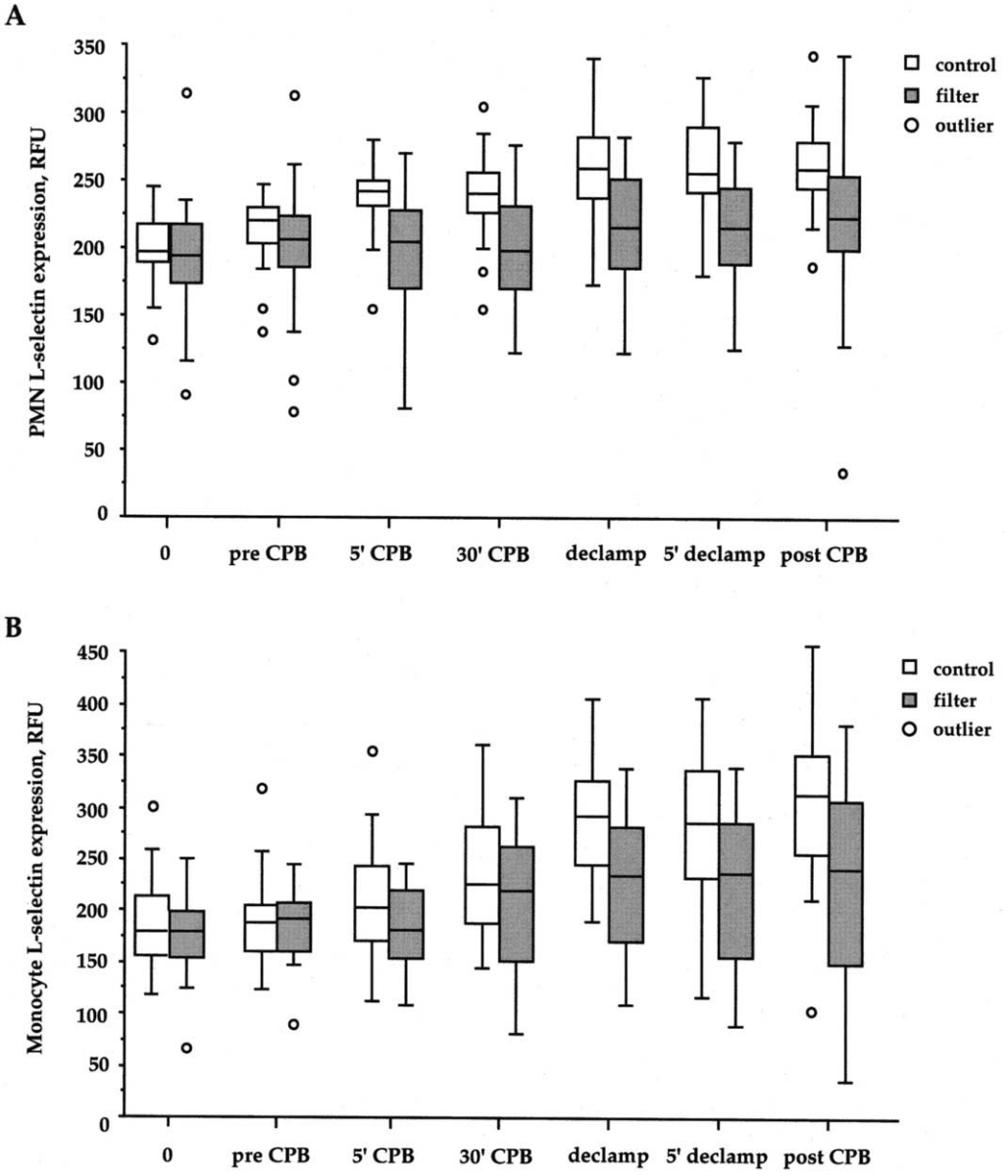

Figure 3. Neutrophil (A) and monocyte (B) L-selectin expressions in control and filter groups. *Boxes* indicate 25th and 75th percentiles and *central lines* indicate median, with *whiskers* representing range. *0,* Before surgery; *pre CPB,* immediately before CPB; *5′ CPB,* 5 minutes of CPB; 30′ CPB, 30 minutes of CPB; *declamp,* immediately before aortic declamping; *5′ declamp,* 5 minutes after aortic declamping, *post CPB,* 5 minutes after CPB. For both neutrophil and monocyte L-selectin expressions, $P < .001$ between groups, 1-way repeated measures ANOVA. Neutrophil L-selectin expression increased significantly in control group (time effect $P < .001$) but did not change significantly with time in filter group ($P = .067$). Monocyte L-selectin expression increased in control group (time effect $P < .001$), but less so in filter group (time effect $P = .014$).

the preoperative level ($P < .001$ between groups, $P < .001$ time effect in control group, $P = .067$ time effect in filter group, 1-way repeated measures ANOVA; Figure 3, *A*). At 5 minutes of CPB, a decrease in neutrophil L-selectin expression occurred across the filter (Table 2).

Preoperative monocyte L-selectin expression was comparable in the groups (Figure 3, *B*). At 5 minutes of CPB, monocyte L-selectin expression was already slightly lower in the filter group, and it remained so during CPB ($P < .001$ between groups, $P < .001$ time effect in control group, $P = .014$ time effect in filter group, 1-way repeated measures ANOVA; Figure 3, *B*). There was no difference in monocyte L-selectin expression across the filter (Table 2).

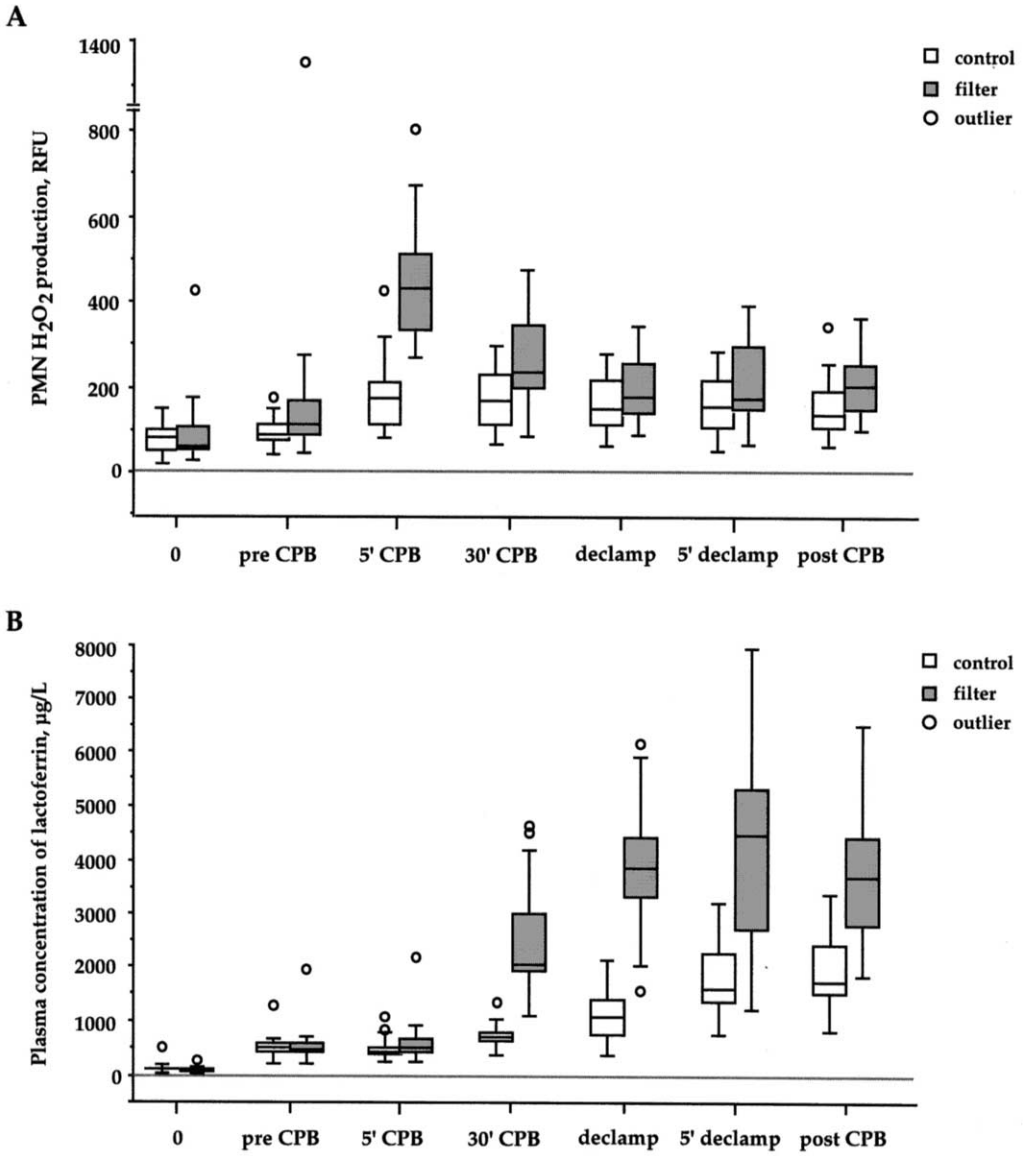

Figure 4. Neutrophil hydrogen peroxide production measured as DCF fluorescence (A) and plasma concentration of lactoferrin (B) in control and filter groups. *Boxes* indicate 25th and 75th percentiles and *central lines* indicate median, with *whiskers* representing range. *0,* Before surgery; *pre CPB,* immediately before CPB; *5′ CPB,* 5 minutes of CPB; 30′ CPB, 30 minutes of CPB; *declamp,* immediately before aortic declamping; *5′ declamp,* 5 minutes after aortic declamping, *post CPB,* 5 minutes after CPB. For both neutrophil hydrogen peroxide production and plasma lactoferrin concentration, $P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA.

Neutrophil Respiratory Burst Activity

Before surgery, neutrophil hydrogen peroxide production, measured as DCF fluorescence, was comparable in the groups (Figure 4, *A*). CPB induced an increase in DCF fluorescence in both groups (Figure 4, *A*). Like CD11b expression, neutrophil DCF fluorescence was already higher in the filter group (median 430 RFU, range 273-804 RFU) than in the control group (median 173 RFU, range 80-433 RFU, $P < .001$, Mann-Whitney *U* test) at 5 minutes of CPB. Thereafter, the DCF fluorescence decreased in the filter group but still remained at a higher level than in the control group ($P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA; Figure 4, *A*). At 5 minutes after aortic declamping, an increase in hydrogen peroxide production occurred across the filter (Table 2).

Plasma Lactoferrin Level

Before surgery, plasma lactoferrin level was comparable in the groups (Figure 4, *B*). CPB induced a time-dependent

increase in lactoferrin level in both groups. In the filter group, however, this increase was steeper ($P < .001$ between groups, $P < .001$ time effect within groups, 1-way repeated measures ANOVA; Figure 4, *B*). There was no difference in plasma levels of lactoferrin across the filter (Table 2).

Discussion

A leukocyte-depleting filter, when used throughout CPB, enhanced activation of circulating phagocytes, measured as increased CD11b and decreased L-selectin expression, and increased hydrogen peroxide production. Importantly, plasma lactoferrin was higher in the filter group. Whereas CD11b and L-selectin expression and hydrogen peroxide production define the activation state of circulating phagocytes, plasma lactoferrin level indicates intravascular neutrophil activation in toto. The rationale for filtering arterial line blood is to remove activated phagocytes from the circulation, thereby decreasing systemic inflammation during cardiac and pulmonary reperfusion. Our findings show that, in terms of phagocyte activation, this goal was not achieved with the leukocyte-depleting filter used.

Although plasma lactoferrin level was clearly elevated in the filter group, it is impossible to determine whether neutrophil activation occurred within the filter, the microvascular bed, or both. At 5 minutes after onset of CPB, neutrophil and monocyte CD11b expression increased and neutrophil L-selectin expression decreased across the filter, indicating that, at least at the beginning of CPB, the filter itself activated neutrophils and monocytes and allowed the activated phagocytes to pass through the filter. Later during CPB, however, no differences were observed across the filter. Neutrophil activation within the filter may have ceased as a result of saturation of the filter.[10,23] Alternatively, activation may have continued, but gradient across the filter was below the detection limit. Indeed, comparison of simultaneously taken blood samples across the filter measures changes only during a single passage of blood through the filter and may be hampered by entrapment of a low number of cells.

Plasma lactoferrin level increased steadily during CPB in the filter group. Because lactoferrin's half-life is only 45 minutes,[24] increasing lactoferrin level is unlikely to have been caused by accumulation of this granular protein in the blood stream. The finding thus reflects ongoing neutrophil activation throughout CPB. Because filter-induced neutrophil activation occurs possibly only at the beginning of CPB, steadily increasing lactoferrin level may originate from neutrophils adhering to the capillary beds. Further, no entrapment of neutrophils was observed in the filter during CPB. Thus lower counts of circulating neutrophils in the filter group may reflect capillary bed sequestration of activated neutrophils. This would also explain the steadily decreasing pattern of CD11b expression and hydrogen peroxide production as activated neutrophils leave the circulation. Theoretically, the filter might therefore increase adherence of circulating neutrophils and potentiate neutrophil-mediated adverse effects of CPB. However, we did not observe any difference in clinical outcome between the groups. All in all, although not harmful, the filter seem ineffective in its task to minimize neutrophil activation.

Chen and colleagues[25] reported that neutrophils in patients undergoing CPB with a filter showed low expression of both CD11b and L-selectin. These results are controversial, however, because low CD11b expression denotes a low activation state, whereas low L-selectin expression denotes a high activation state of the very same neutrophil population. In other words, neutrophils according to one index were activated but according to another were not. Inconsistent results of Chen and colleagues[25] and the discrepancy between their and our CD11b results may be due to differences in sample handling. To prevent increase in CD11b expression ex vivo, Chen and colleagues[25] treated blood samples with formalin solution at 37°C. Such treatment, however, interferes with subsequent leukocyte staining with commercial antibodies,[20] and we therefore preferred rapid sample cooling at 0°C to formalin fixation.

The mechanisms of filter-induced phagocyte activation are unknown. According to the manufacturer, the LG6 filter is composed of a special leukocyte-depleting filtration medium of unannounced composition and polyester fiber elements intended to trap leukocytes, particularly neutrophils. Within the filter, CD11b/CD18 complex is the most probable molecule to mediate neutrophil adherence to filter elements, because it mediates phagocyte adhesion to a variety of foreign bodies.[26,27] Additionally, CD11b/CD18 and L-selectin mediate cell-to-cell contacts, such as phagocyte aggregation.[28] Further, they promote phagocyte activation.[29] The cell-to-cell contacts are reversible events.[30] The filter possibly promotes transient phagocyte adherence, followed by detachment and reentry of activated cells into the circulation. In addition, phagocytes trapped by the filter may release inflammatory mediators, promoting phagocyte tissue sequestration.[1,2] Phagocyte overactivation in the filter group coincided with cardiac and pulmonary reperfusion after aortic declamping. At that critical moment, phagocyte activation is not desirable, because it may enhance myocardial and pulmonary reperfusion injury.

We conclude that the use of the LG6 arterial line leukocyte filter is associated with increased neutrophil and monocyte activation. At least at the beginning of CPB, the increased phagocyte activation is localized in the filter itself. The results give credence to the view that, if not harmful, the LG6 leukocyte-depleting filter is at least ineffective in its principal task of diminishing phagocyte activation during CPB.

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